

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
24 January 2002 (24.01.2002)

PCT

(10) International Publication Number
WO 02/05860 A1

(51) International Patent Classification⁷: A61K 51/04,
31/438, G01N 33/567, C07D 491/107

(21) International Application Number: PCT/US01/21731

(22) International Filing Date: 10 July 2001 (10.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/218,397 14 July 2000 (14.07.2000) US

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(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

Best Available Copy

(54) Title: RADIOLIGAND AND BINDING ASSAY

(57) Abstract: The present invention is directed to the radioligand compound, [35S]-radiolabeled (+)-N-[1 -(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxy-2H-1-benzopyran-2,4 -piperidin]-6-yl]methanesulfonamide. Also within the scope of this invention is a method for identifying compounds that bind to the IKr channel, and may have antiarrhythmic activity.

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TITLE OF THE INVENTION

RADIOLIGAND AND BINDING ASSAY

BACKGROUND OF THE INVENTION

5 The present invention provides for a radioligand compound that is a useful agent to screen for potential Class III antiarrhythmic compounds and method of screening for this I_{K_r} activity using this radioligand compound. In the past, a similar binding assay was conducted using a [^3H]-radioligand capable of binding to ether-a-go-go-related gene (ERG) in guinea pig myocytes or patch clamping on intact cells
10 were carried out. See Geonzon, R. et al. J. Mol. Cell Cardiol. 30, p.1691-1701 (1998); Chadwick, C.C. et al. Circulation Research 72, p.707-714 (1993); Duff, H. J. et al. Circulation Research 77, p.718-725 (1995); and Fiset, C. et al. J. Mol. Cell Cardiol. 28, p.185-1096 (1996).

 The present invention offers several advantages over the previous
15 methods. Use of a [^{35}S]-labeled radioligand offers the advantage that [^{35}S] is a higher energy and specific activity radioisotope than [^3H], allowing for scientists to refine the assay conditions with the use of less radioligand and human ERG (also referred to as hERG) channel, as well as the ability to set up a more sensitive assay. The current binding assay has also been developed using membranes from cells expressing hERG,
20 rather than ERG in intact animal cells, as the source of the ion channel. Thus, the use of human ERG in the present assay will provide investigators with a more relevant binding assay over the animal ERG.

 Additionally, membranes are a more convenient source of the ion channel compared to intact cells. A larger throughput is possible as a large amount of
25 the membrane can be made in advance, stored frozen and thawed on the day of the assay. Also, a membrane source of ion channel has both its extracellular and intracellular sites exposed and is not dependent on changes in the physiology of an intact cell for all potential binding sites to be exposed. A final advantage of the [^{35}S]-based binding assay is that it can be carried out with much higher throughput
30 compared to a whole cell patch clamp assay. Thus, the current invention is more suited to broad-based screening. This assay is used as a counterscreen in research programs where long QT activity is not desirable.

 US Patent No. 5,633,247 discloses and claims the unlabelled compound (also referred to as the cold ligand), N-[1'-(6-cyano-1,2,3,4-tetrahydro-

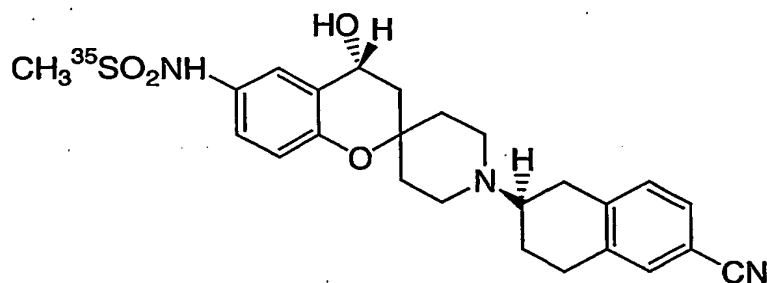
2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide.

SUMMARY OF THE INVENTION

5 This invention relates to a radioligand compound, [^{35}S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide (Formula I) or pharmaceutically acceptable salts, hydrates or solvates thereof. Also within the scope of this invention is a method for identifying compounds that bind to the $\text{I}_{\text{K}_\text{r}}$ channel.

DETAILED DESCRIPTION OF THE INVENTION

A radioligand compound of Formula I



15 or pharmaceutically acceptable salts, hydrates, or solvates thereof. The radioligand compound of Formula I is also referred to as [^{35}S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide. The compound of Formula II is the unlabeled compound, (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide.

25 An embodiment of the invention includes the radioligand compound of Formula I which possesses a specific activity of greater than about 500 Ci/mmol., and preferably in the range of about 900 Ci/mmol. to about 1498 Ci/mmol.

A method of characterizing an ion channel as an $\text{I}_{\text{K}_\text{r}}$ channel comprising contacting the ion channel with the radioligand compound, [^{35}S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-

4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide, and determining if the radioligand compound binds to the ion channel.

A method for characterizing the activity of a compound as an I_{K_r} channel blocker comprising contacting the test compound with a membrane containing the I_{K_r} channel in the presence of the radioligand compound, [35S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide, and monitoring whether the test compound influences the binding of the radioligand compound to the membrane containing the I_{K_r} channel. The membrane containing the I_{K_r} channel is derived from an appropriate cell line capable of being stably transfected with the ERG gene and expressing the I_{K_r} channel protein, representative examples of the same are the HEK 293 cells (ATCC No. CRL-1573, American Type Culture Collection 10801 University Boulevard, Manassas, VA 20110) or CHO cells that have been stably transfected with the human, canine or primate ERG gene and are capable of expressing the I_{K_r} channel protein. The human ERG gene is disclosed in T. Itoh, et al. Human Genetics 102(4), pp. 435-439 (1998) and M.D. Adams, et al. Nature 377:3-174 (1995). The radioligand compound useful in this method possesses a specific activity of greater than 500 Ci/mmol., and preferably in the range of about 900 Ci/mmol. to about 1498 Ci/mmol.

A method for assessing the binding of a test compound to a membrane containing the I_{K_r} channel using a radioligand of Formula I, [35S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide, comprising the steps of:

- 1) preparing solutions of the test compound at 5 or more different concentrations, a solution of control vehicle and a solution of (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide (compound of Formula II) in a solvent;
- 2) mixing the radioligand compound of Formula I with the membrane containing the I_{K_r} channel diluted with an assay buffer to form a membrane/radioligand mixture of known concentration;
- 3) incubating a quantity of known concentration of the membrane/radioligand mixture with the solution of test compound, control vehicle or compound of Formula II, as recited in Step 1, for a set time period at a

temperature range of between about 40°C and about 37°C to give a mixture of membrane bound with the radioligand and the test compound, the control vehicle or the compound of Formula II, where the final concentration of the membrane containing the I_{K_r} channel is predetermined;

- 4) isolating from the incubated mixture the membrane bound with the radioligand and the test compound, the control vehicle or the compound of Formula II;
- 5) measuring the radioactivity of the isolated membrane bound with the radioligand and the test compound, the control vehicle or the compound of Formula II;
- 6) repeating steps 3 through 5 with the test compound at each concentration, the solution of control vehicle and the solution of the compound of Formula II, as recited in Step 1; and
- 7) calculating the IC_{50} corresponding to the measured radioactivity of: 1) the membrane bound with the radioligand and each concentration of the test compound, 2) the membrane bound with the radioligand and with the control vehicle, and 3) the membrane bound with the radioligand and the compound of Formula II.

The potency of compound interaction with the I_{K_r} channel or IC_{50} , is determined by comparing the [^{35}S]-radioligand counts bound to the membranes in the presence of a solvent vehicle (100% control), in the presence of 1 μM Formula II (final non-specific binding) and at each of seven different concentrations of the test compound. The number of different concentrations tested can be more or less than seven. A 100 μM solution of the test compound in a solvent, such as dimethylsulfoxide (DMSO), methanol, water or buffer, is prepared and seven concentrations of the test compound are prepared by through either log (10-fold) dilutions, 5-fold dilutions or 3-fold dilutions of the stock 100 μM solution of test compound. The percent control value is determined for each condition and a non-linear 4 parameter regression plot is made and the inflection point or IC_{50} , of this plot is determined. This value is reported as a measure of the compound's affinity of interaction with the ERG channel. When the data do not adequately fit the 4 parameter equation either the maximum or minimum binding, or both, are fixed and either a 3 or 2 parameter fit, respectively, are used to generate the inflection point.

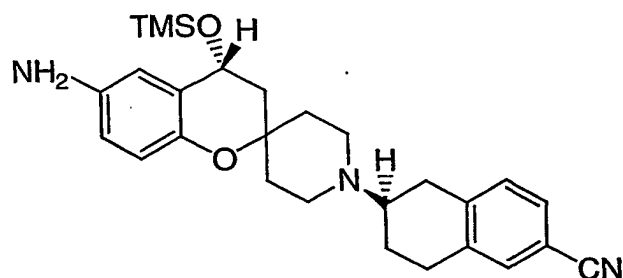
The membrane-bound radioligand is monitored after isolation by either filtration from the free radioligand using for example the Unifilter (Packard), or by using alternate techniques that include the Scintillation Proximity Assay (SPA) beads (Amersham Pharmacia Biotech) or the Flashplates (New England Nuclear Life Sciences). Use of the latter two techniques requires the incubation of the membranes, radioligand and compound with either the SPA bead in a standard assay plate or in a Flashplate.

A method for assessing the binding of a test compound to a membrane containing the I_{K_r} channel using a radioligand of Formula I, [^{35}S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide, comprising the steps of:

- 1) preparing assay wells with 4 μl of the test compound in dimethylsulfoxide (DMSO) diluted 100x with assay buffer at 5 or more different concentrations, a control vehicle of DMSO and a DMSO solution of (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide (compound of Formula II);
- 2) adding the radioligand compound of Formula I at 50 pM to the membrane containing the I_{K_r} channel diluted with assay buffer to form a membrane/radioligand mixture;
- 3) incubating each assay well with 400 μl of the 50 pM membrane/radioligand mixture for about 75 minutes to about 90 minutes at room temperature (25°C) to give assay wells containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II where the final concentration of the membrane containing the I_{K_r} channel is 11 $\mu\text{g/ml}$;
- 4) filtering the incubated assay wells through 0.1% BSA presoaked filters to isolate on the filters the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II;
- 5) washing each of the filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II about 5 times with 500 μl of ice cold wash buffer;

- 6) drying the washed-filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II at room temperature in a fume hood;
- 5 7) adding 50 μ l Microscint-20 microscintillate to the dried-filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II;
- 8) measuring the microscintillation count of the microscintillation-treated filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II for one minute; and
- 10 9) calculating the IC₅₀ corresponding to the measured microscintillation count of: 1) the microscintillation-treated filters containing the membrane bound with the radioligand and each concentration of the test compound, 2) the microscintillation-treated filters containing the membrane bound with the radioligand and with the control vehicle, and 3) the microscintillation-treated filters containing the membrane bound with the radioligand and the compound of Formula II.
- 15
- 20 The method as recited above, wherein the membrane containing the I_{Kr} channel is derived from a cell line transfected with the ERG gene, wherein the cell line is HEK 293 cells or CHO cells, and preferably HEK 293 cells and wherein the ERG gene is human, canine or primate and preferably human or canine.
- 25 The method as recited above, wherein the solutions of the test compound are prepared in Step 1 at 7 different concentrations (dilutions).
- The method as recited above, wherein the radioligand compound of Formula I possesses a specific activity of in the range of greater than 500 and preferably in the range of about 900 Ci/mmol. to about 1498 Ci/mmol. The method as recited above, wherein the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II is filtered in Step 4 with Unifilters (Parkard).
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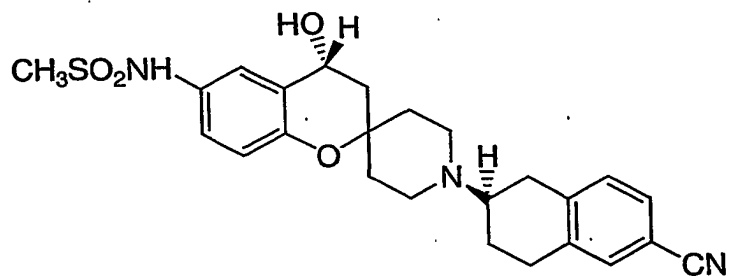
An embodiment of the invention is the process for the preparation of



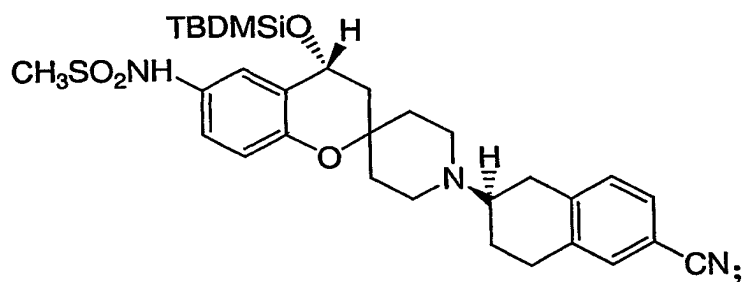
a key intermediate in the preparation of the radioligand compound of Formula I, which comprises the steps of

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(1) reacting the alcohol with 2,6-lutidine and t-butyldimethylsilyl triflate

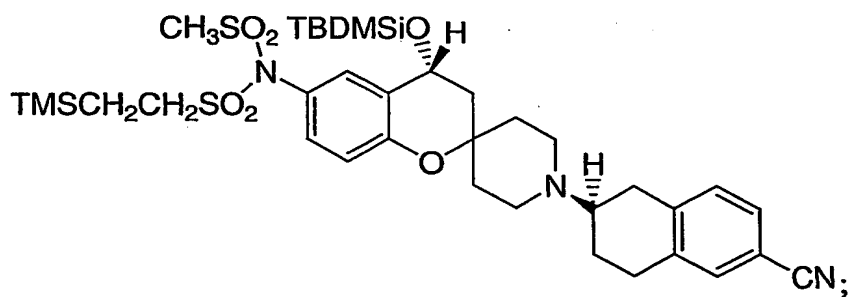


to give a t-butyldimethylsilyl-protected hydroxyl compound

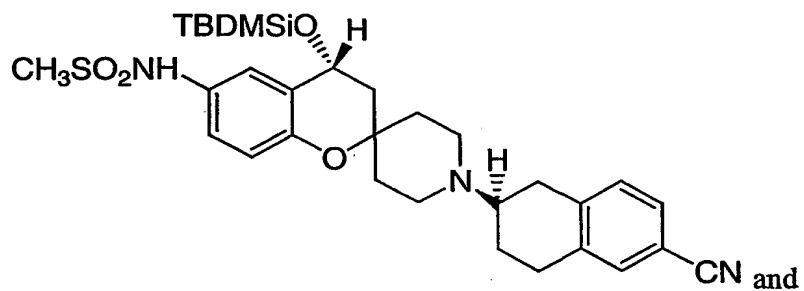


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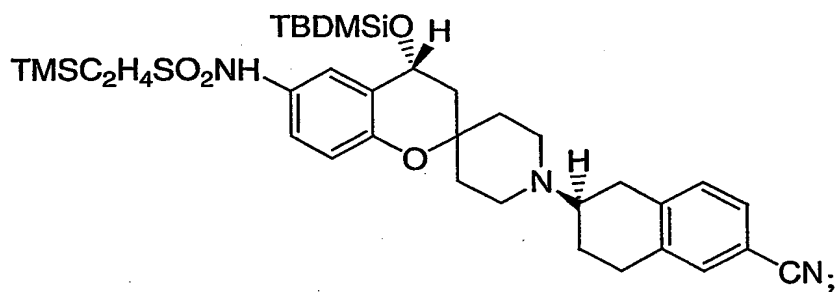
(2) alkylating the t-butyldimethylsilyl-protected hydroxyl compound by treating with sodium hydride, and then treating with 2-trimethylsilylethanesulfonylchloride to give the disubstituted sulfonamide compound



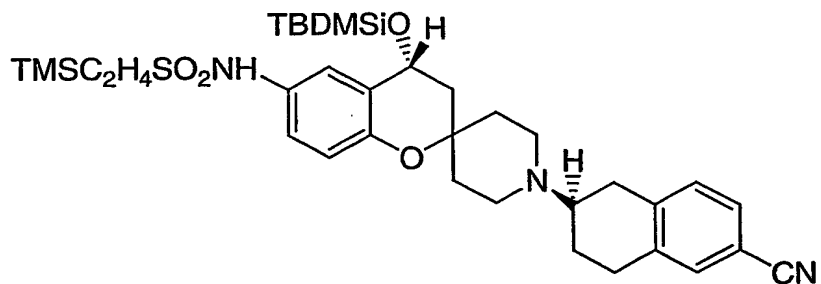
(3) treating the disulfonamide with an C₁-C₈-alkanethiolate to give a mixture of the following sulfonamides;



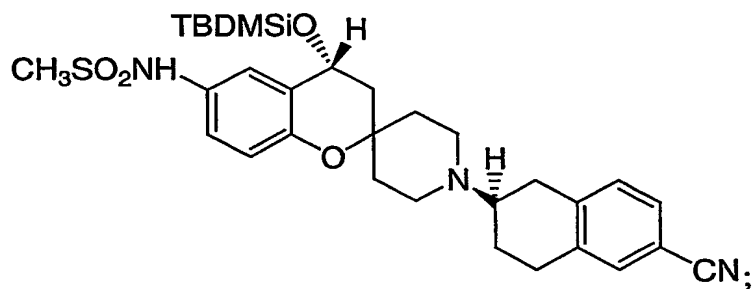
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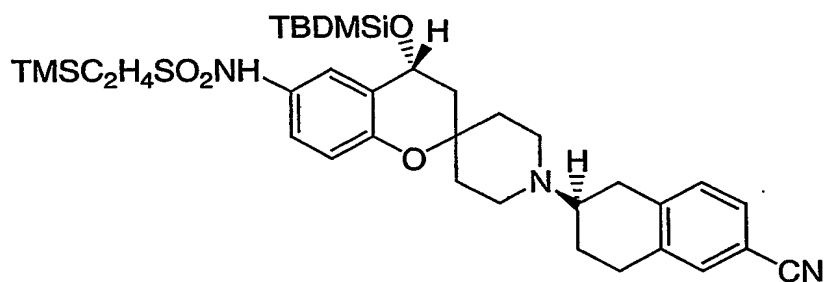
(4) separating the sulfonamide mixture using chromatography to isolate the non-polar sulfonamide eluting with a non-polar solvent system



and then eluting off the polar isomer using a polar solvent system

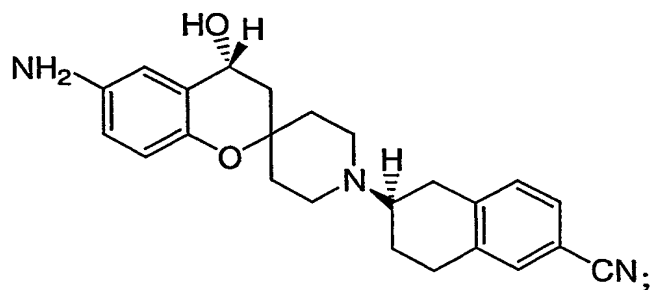


(5) desulfonating the non-polar isomer

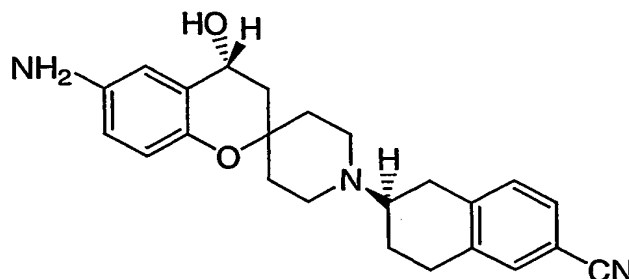


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using a fluoride compound in an organic solvent and heating for about 24 hours to about 48 hours to give the free alcohol-amine



(6) reacting the free alcohol-amine with trimethylsilylimidazole in an organic solvent



5 to give the desired trimethylsilyloxy compound.

The starting material (also referred to as the compound of Formula II) for the process as recited above is described in Patent Nos. 5,633,247, 5,206,240, 5,484,923 and 5,464,762. Additionally, an asymmetric synthesis of the tetrahydronaphthalene intermediate in the synthesis of this starting material is described

10 in British Publication No. 2,290,790 published on January 10, 1996. The process as recited above, wherein the alkylation in step 2 is stirred at room temperature for up to 24 hours, and preferably for 18 hours. The process as recited above, wherein the C1-C8-alkanethiolate used in step 3 is a branched or straight chain alkanethiolate, such as sodium or potassium hexanethiolate, sodium or potassium butanethiolate, sodium or

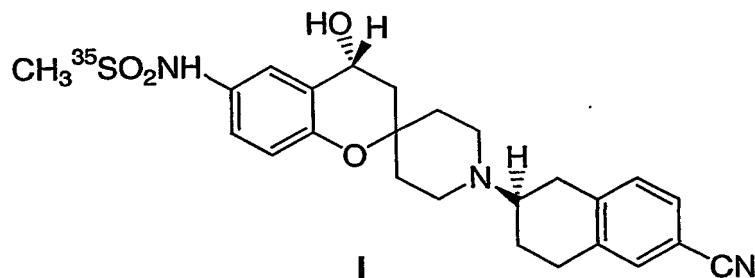
15 potassium 2-methylpentanethiolate, sodium or potassium 2-methylpropanethiolate, sodium or potassium methanethiolate, sodium or potassium ethanethiolate and preferably sodium 2-methylpropanethiolate. The process as recited above, wherein the desulfonylation reaction in step 3 is run for less than 24 hours, preferably for about 30 minutes to one hour. The process as recited above, wherein the separation of the sulfonamide mixture in step 4 is run using flash chromatography with a solvent

20 system of ethyl acetate and hexane to ethyl acetate and methanol, and preferably with a non-polar solvent system of 1:1 ethyl acetate: hexane and a polar solvent system of 99:1 ethyl acetate: methanol. The process as recited above, wherein the fluoride compound used in the desulfonylation of step 5 is selected from: cesium fluoride (CsF), and tetrabutylammonium fluoride, and preferably CsF. The process as recited

25 above, wherein the organic solvent used in the desulfonylation of step 5 is selected from: dimethylformamide (DMF), dimethylsulfoxide, and N-methylpyrrolidinone, and

is preferably DMF. The process as recited above, wherein the organic solvent in step 6 is acetonitrile, tetrahydrofuran, or ether, and preferably is acetonitrile.

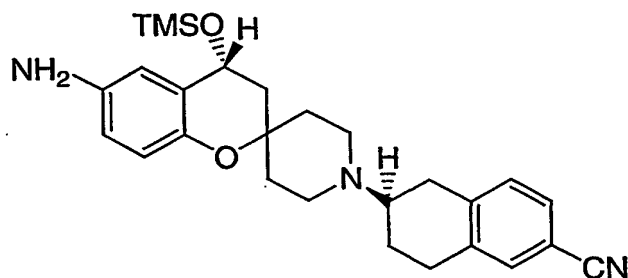
An embodiment of the invention is a process for the preparation of a radioligand compound of Formula I



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comprising the steps of:

(a) reacting the amine



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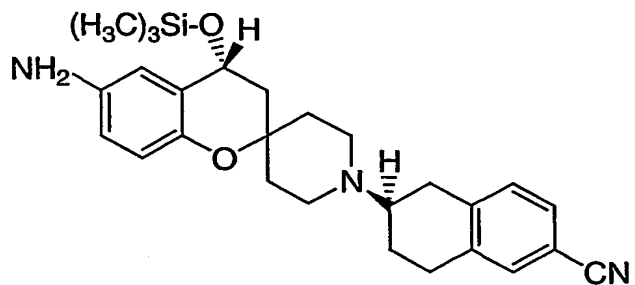
with [^{35}S]-methanesulfonyl chloride in the presence of an organic base to form the silyl-protected [^{35}S]-methanesulfonamide; and

(b) removing the silyl-protecting group of the silyl-protected [^{35}S]-methanesulfonamide with trifluoroacetic acid to give the radioligand compound of Formula I.

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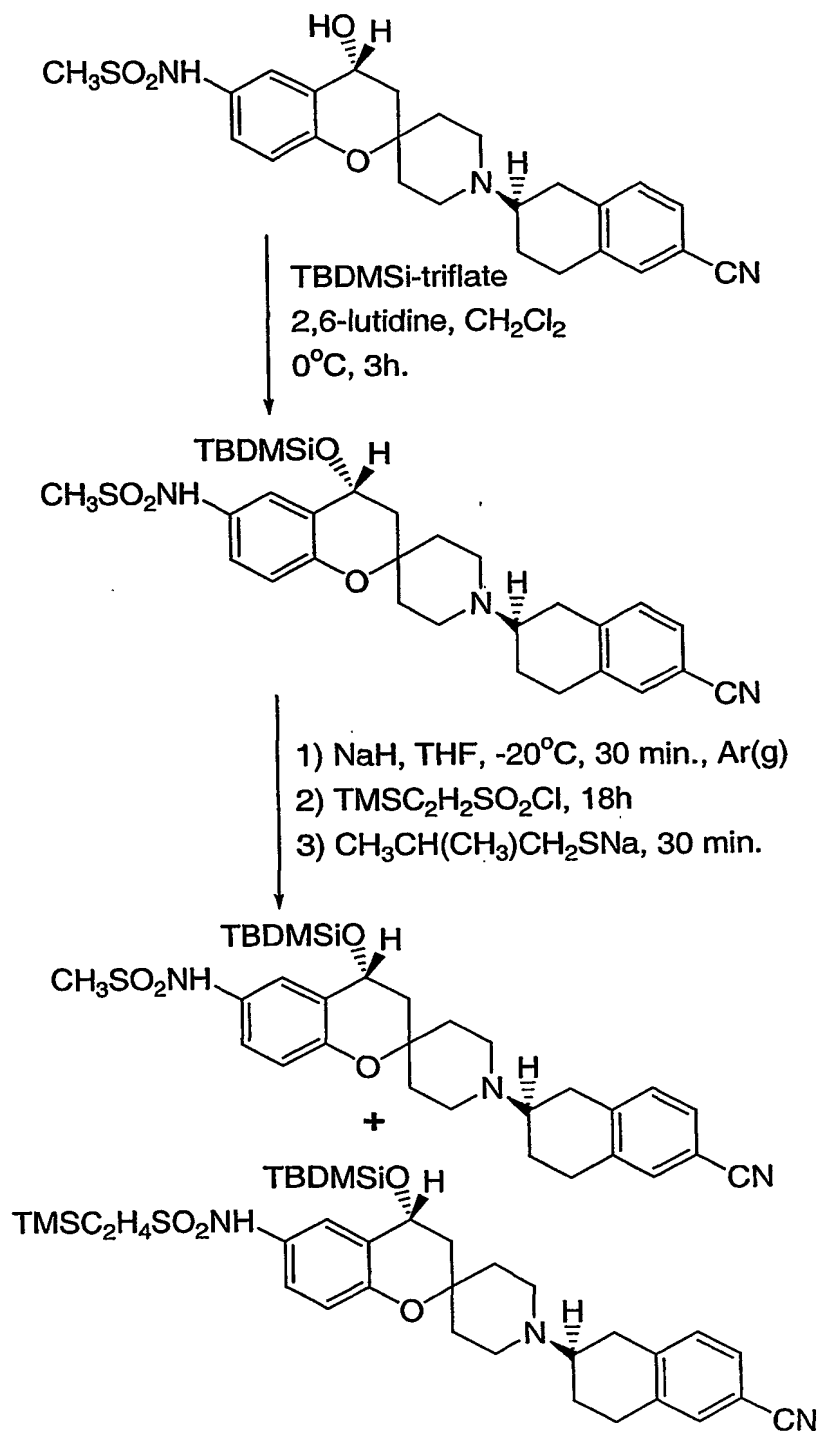
The process, wherein an organic base such as triethylamine, trimethylamine, and diisopropylethylamine is used, preferably triethylamine. The process is preferably run at room temperature for about 2 hours under anhydrous conditions.

Also within the scope of the invention is the key intermediate in the synthesis of the radioligand compound of Formula I, which is the amine compound,

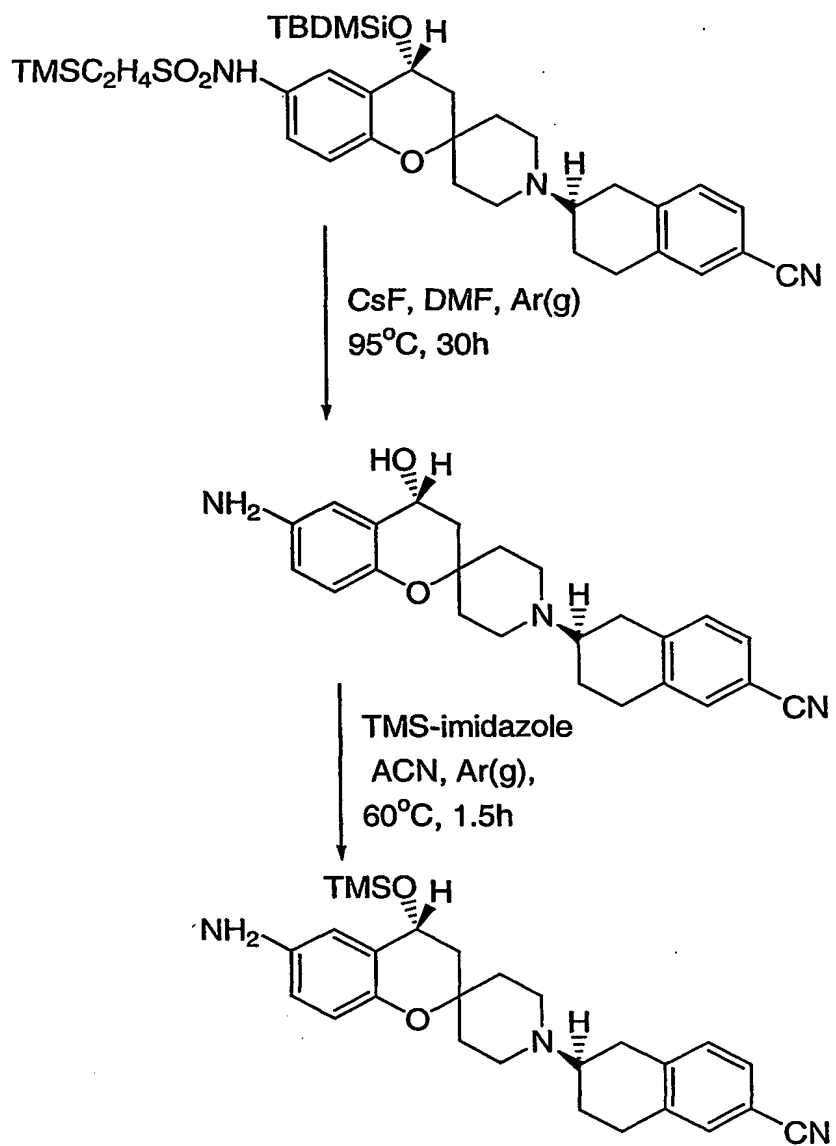


- The radioligand compound [^{35}S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide is prepared utilizing the following synthetic route. Schemes 1 and 2 describe the steps for preparing N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-trimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]amine from
- 5 N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide. Scheme 3 describes preparation of the radiolabeled methanesulfonyl chloride, using sodium [^{35}S]-sulfite to prepare sodium [^{35}S]-methanesulfonate which is then converted to the sulfonyl chloride, and the sulfonylation of the amine with the radiolabeled
- 10 methanesulfonyl chloride, followed by the removal of the trimethylsilyl protecting group.
- 15

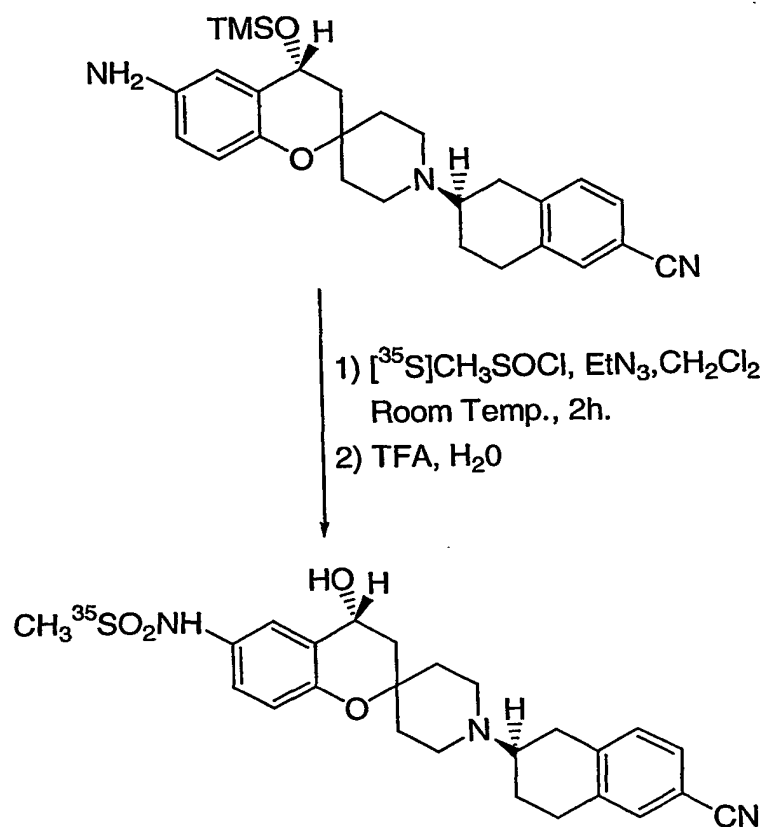
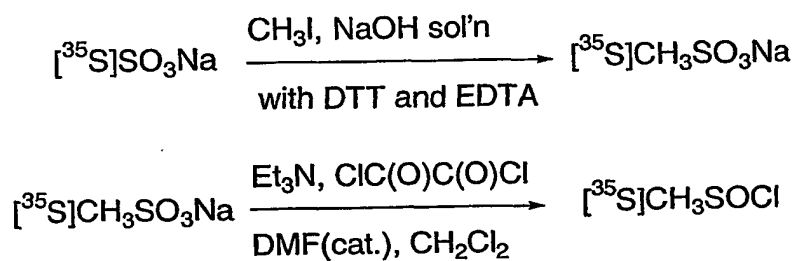
SCHEME1



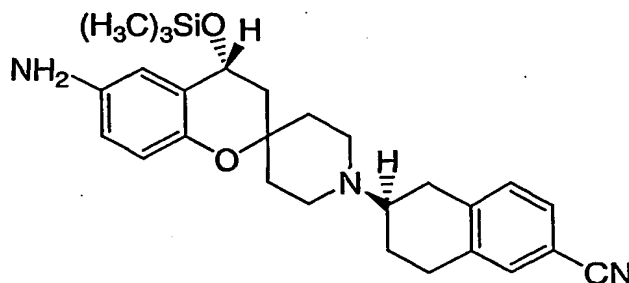
SCHEME 2



SCHEME 3



The following examples illustrate this invention, and as such are not to be considered as limiting the invention set forth in the claims appended hereto.

EXAMPLE 1

5 N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-
trimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]amine

Step A: N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-
4(R)- t-butyltrimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-
yl]methanesulfonamide

10 To a -20°C solution of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-
naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-
yl]methanesulfonamide (5.0 gm, 10.7 mmol) in dichloromethane (50 mL) under argon
was added 2,6-lutidine (3.74 mL, 32.1 mmole) followed by t-butyltrimethylsilyl
triflate (6.46 mL, 26.8 mmole). The reaction mixture was allowed to stir at 0°C for 3
15 hrs. until complete. The reaction was concentrated in vacuo, diluted with methanol
and 1N sodium hydroxide slowly added (110 mL). The mixture was stirred for 30
min. extracted with ethyl acetate (2 X 200mL), dried over sodium sulfate and
concentrated in vacuo to an oil (9 gm). Purification by flash chromatography using
95/5 dichloromethane/acetone followed by 90/10/3 dichloromethane/ acetone/
20 methanol gave upon concentration N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-
naphthalenyl)-3,4-dihydro-4(R)- t-butyltrimethylsilylhydroxyspiro[2H-1-benzopyran-
2,4'-piperidin]-6-yl]methanesulfonamide (5.5 gm, 88%) as a white solid. MS m/z 582
(M^{+}).

Step B: Preparation of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)- t-butyl dimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]trimethylsilylethanesulfonamide

To a solution of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)- t-butyl dimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide (2.5 gm, 4.30 mmol) in tetrahydrofuran under argon was added sodium hydride (60 % disp., 0.50 gm, 13.0 mmole). The mixture was allowed to stir for 30 min. cooled to -20 °C and trimethylsilylethanesulfonyl chloride (2.5 mL, 12.4 mmole) added. The reaction was allowed to stir for 18 hrs., after which sodium 2-methylpropanethiolate (5.0 gm, 44.0 mmole) was added in one portion. The reaction was allowed to stir for 30 min. diluted with water (200 mL) and extracted with ethyl acetate (400 mL). The organic extract was washed with brine, dried over sodium sulfate, concentrated in vacuo to an oil and purified by flash chromatography using 1/1 ethyl acetate/hexane to give N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)- t-butyl dimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]trimethylsilylethanesulfonamide (1.43 gm, 50%), followed by 99/1 ethyl acetate/methanol to give N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)- t-butyl dimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide (1.1 gm, 44%). MS m/z 668 (M⁺).

Step C: Preparation of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-amine

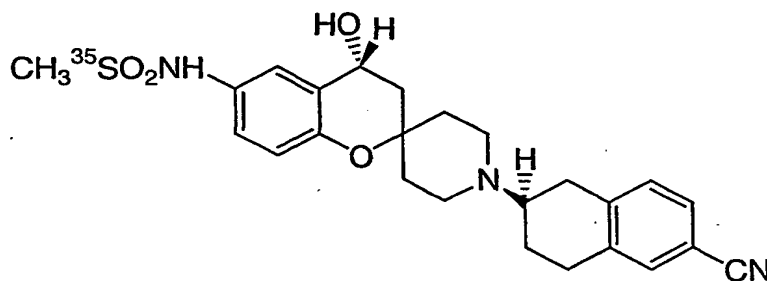
To a mixture of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)- t-butyl dimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]trimethylsilylethanesulfonamide (0.50 gm, 0.75 mmole) in dimethylformamide (5 mL) under argon was added cesium fluoride (0.34 gm, 2.25 mmole) and the mixture heated to 95°C for 30 hrs. The reaction was cooled diluted with 10 % aqueous sodium bicarbonate (50 mL) and extracted with ethyl acetate (2 X 50 mL). The organic extracts were dried over sodium sulfate concentrated in vacuo to an oil and purified by flash chromatography using 90/10 dichloromethane/methanol to give N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]amine, (0.218 gm, 80 %) as a foam. MS m/z 390 (M⁺), ¹H NMR (CDCl₃) δ 1.4-2.2 (9 H, m), 2.6-3.2 (9 H, m), 3.4 (2 H, br s),

4.82 (1 H, br t), 6.60 (1 H, dd, J=9.3 and 2.8 Hz), 6.71 (1 H, d, J= 9.3 Hz), 6.81 (1 H, d, J=2.8 Hz), 7.18 (1 H, d, J=9.3 Hz), 7.37 (2 H, m).

Step D: Preparation of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-trimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]amine

To a solution of N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]amine (50 mg, 0.128 mmole) in acetonitrile under argon was added trimethylsilylimidazole (0.36 gm, 2.5 mmole) and the reaction warmed to 60 C for 1.5 hrs. The reaction was concentrated in vacuo and purified by flash chromatography using 95/5/0.1 dichloromethane/methanol/triethylamine to give N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-trimethylsilyloxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]amine as a foam (58 mg, 98 %). MS m/z 462 (M⁺), ¹H NMR (CDCl₃) δ 0.09 (9 H, s), 1.4-2.1 (8 H, m), 2.4-2.9 (9 H, m), 3.2 (2 H, br s), 4.65 (1 H, t), 6.41 (1 H, dd, J=9.2 and 3.3 Hz), 6.49 (1 H, d, J= 3.3 Hz), 6.52 (1 H, d, J=9.2 Hz), 7.03 (1 H, d, J=9.2 Hz), 7.22 (2 H, m).

EXAMPLE 2



[³⁵S]-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide

Step A: Sodium [³⁵S]-methanesulfonate

To a solution of sodium [³⁵S]sulfite (1 Ci/mmol, 215 μCi in 100 μL of 0.1N NaOH solution with 10 mM DTT and 1 mM EDTA) (which may be obtained by reduction of [³⁵S]sulfuric acid on a copper surface with trapping of the generated

[³⁵S]sulfur dioxide in sodium hydroxide solution) was added 25 μ L of methyl iodide and the reaction was stirred at room temperature for 20 min. Analysis of radioactivity by HPLC (Astec cyclobond I column, gradient of 10 mM nitric acid solution to 180 mM nitric acid solution over 20 min, 1 mL/min) indicated complete conversion to
5 [³⁵S]-methanesulfonic acid. The solution was concentrated under nitrogen to near dryness, 500 μ L of DI water was added, and the solution was degassed with nitrogen. The resulting solution of sodium [³⁵S]-methanesulfonate (177 μ Ci) was stored at 0°C.

Step B: [³⁵S]-Methanesulfonyl chloride

10 A solution of [³⁵S]-methanesulfonic acid (3.37 mCi in 2.08 g of 0.1 N sodium hydroxide, specific activity = 1342 Ci/mmol, approx 90% RCP, obtained from New England Nuclear) was blown to dryness under a stream of nitrogen at 50°C. To the resulting residue was added dichloromethane (1 mL) followed by oxalyl
15 chloride (100 μ L, 0.54 mmol). Vigorous gas evolution occurred for about 1.5 hr, at which point addition of additional oxalyl chloride (20 μ L) did not produce any visible reaction. A 10% solution of dimethylformamide in dichloromethane (20 μ L) was added which resulted in a change in color to greenish yellow. Addition of oxalyl
20 chloride (20 μ L, 0.761 mmol total) did not produce visible reaction and the reaction mixture was stirred at 0°C for 12 hr. The mixture was allowed to warm to room temperature, diluted with dichloromethane (3 mL), counted (2.37 mCi), and washed
25 with 5% sodium bicarbonate solution (2 X 1 mL) followed by 2% sodium bisulfite solution (1 mL). The dichloromethane solution was dried over sodium sulfate and stored in dry CH₂Cl₂ at -78 °C in a desiccator. HPLC: Zorbax SB-C8 4.8 x 250 mm analytical column, 80% water (containing 0.1% trifluoroacetic acid) : 20% acetonitrile
to 20% water(containing 0.1% trifluoroacetic acid) : 80% acetonitrile in 30 min., UV = 254 nm, Flow rate = 1 ml/min, Retention time (Rt) of [³⁵S]-methanesulfonyl
chloride = 9.2 min. Measurement of the radioactivity by counting the resulting
solution indicated 1.75 mCi (approx 58% yield) to be present. The details for the
preparation of the [³⁵S]-methanesulfonyl chloride is described in "Development of a
30 High Specific Activity Sulfur-35 Labelled Sulfonamide Radioligand That Allowed the Identification of a New Growth Hormone Secretagogue Receptor." Dean, D.C., Nargund, R.P., Melillo, D. G., Pong, S.S., Patchett, A.A., Smith, R.G, Chaung, L.P., Ellsworth, R.L., Griffin, P., Van der Ploeg, L. *J. Med. Chem.* 1996, 39, 1767.

Step C: Preparation of [³⁵S]-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]methanesulfonamide

From a stock solution of [³⁵S]-methanesulfonyl chloride in CH₂Cl₂ (freshly dried over anhydrous sodium sulfate), 25 mCi of [³⁵S]-methanesulfonyl chloride was transferred into a 25 ml pear shaped flask and excess solvent was distilled at atmospheric pressure at 60-64 °C. The distillation was stopped when the volume of solution was about 100 -200 µl. The flask was removed from the heating bath and allowed to cool slightly. This solution was then transferred via blunt-end syringe to a solution containing amine product of Example 1, Step D (~5 mg, ~5000 fold excess relative to the [³⁵S]-methanesulfonyl chloride) in 30 µl of anhydrous CH₂Cl₂. The distillation flask was rinsed with anhydrous CH₂Cl₂ (2 x 50 µl) and combining organic solvent was transferred to the reaction mixture. To this solution, was added 10 µl of anhydrous triethylamine (stored under activated molecular sieves 4A). The reaction mixture was stirred at room temperature for 2 hr. The reaction mixture was assayed by HPLC analysis by quenching a aliquot of the reaction solution (<0.1 µL) into HPLC eluent (HPLC system: Zorbax-SB-C8 4.5 x 250 mm analytical column, 70 % water (containing 0.1% HClO₄): 30 % acetonitrile for 30 min to 100% MeCN over 10 min, then hold for 10 min, UV = 230 nm, flow rate = 1 ml /min, Rt of [³⁵S]MeSO₃H = 3.1 min , Rt of amine 1 = 5.1 min, Rt of [³⁵S]sulfonamide was 10.1 min, ~50% [³⁵S]MeSO₃H, 45% radiochemical yield).

The reaction was diluted with 200 µl of water and 10 µl of trifluoroacetic acid (in order to deprotect the O-trimethylsilyl group). After stirring for 30 min at room temperature, the reaction mixture was diluted with 5 ml CH₂Cl₂ and stirred vigorously. The dichloromethane layer was separated and washed with saturated sodium bicarbonate (2 x 2 ml). The aqueous layer was extracted with CH₂Cl₂ (2 x 10 ml). The combined organic layer was dried over anhydrous sodium sulfate and evaporated to dryness using a rotary evaporator under reduced pressure. The resulting residue was dissolved in a mixture of acetonitrile:water (2:1 v:v) and purified using preparative HPLC (Zorbax-SB-C8 9.4 x 250 mm semi prep column, 75% water containing 0.1% HClO₄ : 25% acetonitrile, UV = 230 nm, flow rate = 3 ml /min,. R_T of MK-0499 = 14.9 min.). The product-containing fractions were combined and neutralized with saturated sodium bicarbonate. Acetonitrile was removed by concentration under nitrogen in a polypropylene container and the resulting solution was passed through a Sep-Pak (Waters, C-18) cartridge. The

cartridge was washed with water (10 ml), followed elution of the product with methanol (2 x 5 ml). The methanol fraction was collected and determined to have a total activity of 11.5 mCi.

5 This batch showed a 1.3% radiochemical impurity at 2.3 min and 1.1 % impurity at 6.7 min. Therefore, this batch was again repurified using Zorbax -SB phenyl semi prep column using same conditions as above, followed by Sep-Pak purification. The total activity obtained was 8.832 mCi with radiochemical purity >99%. The compound of Formula I has a specific activity of 1368 Ci/mmol.

10

EXAMPLE 3

[³⁵S] MK499 binding to hERG K⁺ channel in HEK 293 cell membranes

Step A: Materials

15 A. Buffers:

1. Assay buffer (pH 7.4, room temp):

	KCl	60 mM
	NaCl	71.5 mM
	CaCl ₂ x 2H ₂ O	1 mM
20	MgCl ₂ x 6H ₂ O	2 mM
	Hepes	10 mM

2. Wash buffer (pH 7.4 at 4°C):

	NaCl	131.5 mM
25	CaCl ₂ x 2H ₂ O	1 mM
	MgCl ₂ x 6H ₂ O	2 mM
	Hepes	10 mM

B. Membranes:

30 Human embryonic kidney cells constitutively expressing hERG are harvested, homogenized in Tris-EDTA buffer (containing 50 mM Tris and 1 mM EDTA (pH 7.4)) and centrifuged at 45,000xg for 20 min. at 4°C. The pellet is resuspended in Tris-EDTA buffer, re-homogenized and centrifuged as above. Finally, the pellet is resuspended in Tris-EDTA buffer, aliquoted and stored at -70°C.

Membranes are briefly homogenized on the day of experiment and each aliquot is used only once.

C. Radioligand: Formula I at a final concentration of 50 pM

5

D. Non-specific displacer: Formula II; 1 μ M

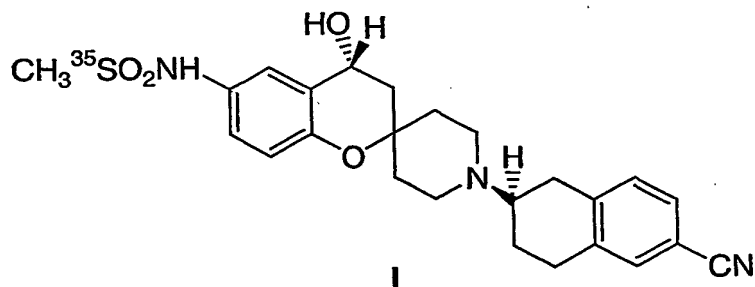
E. Cells: Cells are HEK 293 stably transfected with hERG cDNA. See Z. Zhou, et al. Biophysical Journal 74 pp. 230-241 (1998). They are in frozen medium. They should
10 be cultured in minimum essential medium (MEM) supplemented with Earle's salts, nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), penicillin (50 units/ml), streptomycin (50 μ g/ml), geneticin (G418, Gibco BRL, 0.4 mg/ml) and fetal bovine serum (10%).

15 Step B: Binding assay

Human ERG (hERG) membranes are diluted into assay buffer, mixed with radioligand to final concentration 50 pM and 400 μ l of membrane/ligand mixture is added per well to 96 well assay blocks (Costar, Cat.# 3957), containing 4 μ l of 100x
20 stocks of tested drugs or 100% DMSO (max. binding) or 100 μ M solution of compound of Formula II (non-specific binding). The final membrane protein concentration in the assay is 11 μ g/ml. This mixture is incubated at room temperature for 75 min., then filtered through GF/B Unifilters (Packard) presoaked in 0.1% BSA and washed 5 x 500 μ l with ice-cold wash buffer. The filters are dried inside the fume
25 hood at room temperature, 50 μ l Microscint-20 is added to each well and Unifilters are counted for 1 min. in Topcount (Packard).

WHAT IS CLAIMED IS:

1. A radioligand compound of Formula I which is



- 5 or pharmaceutically acceptable salts thereof.

2. The radioligand compound of Formula I, as recited in Claim 1, which possesses a specific activity of greater than 500 Ci/mmol.

- 10 3. The radioligand compound of Formula I, as recited in Claim 1, which possesses a specific activity of in the range of about 900 Ci/mmol. to about 1498 Ci/mmol.

- 15 4. A method of characterizing an ion channel as an I_{K_r} channel comprising contacting the ion channel with the radioligand compound of Claim 1 and determining if the radioligand compound binds to the ion channel.

- 20 5. A method for characterizing the activity of a compound as an I_{K_r} channel blocker comprising contacting the test compound with a membrane containing the I_{K_r} channel in the presence of the radioligand compound of Claim 1 and monitoring whether the test compound influences the binding of the radioligand compound to the membrane containing the I_{K_r} channel.

- 25 6. The method as recited in Claim 5, wherein the membrane containing the I_{K_r} channel is derived from a cell line transfected with the ERG gene.

7. The method as recited in Claim 6, wherein the cell line is HEK 293 cells or CHO cells.

8. The method as recited in Claim 7, wherein the ERG gene is human, canine or primate.

5 9. The method as recited in Claim 8, wherein the radioligand compound of Formula I possesses a specific activity of in the range of about 900 Ci/mmol. to about 1498 Ci/mmol.

10 10. A method for assessing the binding of a test compound to a membrane containing the I_{K_R} channel using a radioligand compound of Formula I, $[^{35}\text{S}]$ -radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide, comprising the steps of:

- 15 1) preparing solutions of the test compound at 5 or more different concentrations, a solution of control vehicle and a solution of (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxyspiro[2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide (compound of Formula II) in a solvent;
- 20 2) mixing the radioligand compound of Formula I with the membrane containing the I_{K_R} channel diluted with an assay buffer to form a membrane/radioligand mixture of known concentration;
- 25 3) incubating a quantity of known concentration of the membrane/radioligand mixture with the solution of test compound, control vehicle or compound of Formula II, as recited in Step 1, for a set time period at a temperature range of between about 40°C and about 37°C to give a mixture of membrane bound with the radioligand and the test compound, the control vehicle or the compound of Formula II, where the final concentration of the membrane containing the I_{K_R} channel is predetermined;
- 30 4) isolating from the incubated mixture the membrane bound with the radioligand and the test compound, the control vehicle or the compound of Formula II;
- 35 5) measuring the radioactivity of the isolated membrane bound with the radioligand and the test compound, the control vehicle or the compound of Formula II;

- 6) repeating steps 3 through 5 with the test compound at each concentration, the solution of control vehicle and the solution of the compound of Formula II, as recited in Step 1; and
- 7) calculating the IC₅₀ corresponding to the measured radioactivity of: 1) the membrane bound with the radioligand and each concentration of the test compound, 2) the membrane bound with the radioligand and with the control vehicle, and 3) the membrane bound with the radioligand and the compound of Formula II.
11. The method as recited in Claim 10, wherein the membrane containing the I_{Kr} channel is derived from a cell line transfected with the ERG gene.
12. The method as recited in Claim 11, wherein the cell line is HEK 293 cells or CHO cells.
13. The method as recited in Claim 12, wherein the ERG gene is human, canine or primate.
14. The method as recited in Claim 13, wherein the solutions of the test compound are prepared in Step 1 at 7 different concentrations.
15. The method as recited in Claim 14, wherein the time period for incubation in Step 3, is about 30 minutes to 1 hour.
16. The method as recited in Claim 15, wherein the temperature for the incubation in Step 3, is room temperature (25°C).
17. The method as recited in Claim 16, wherein the membrane-bound with radioligand or test compound is isolated in Step 4 with Unifilters, Scintillation Proximity Assay (SPA) beads or the Flashplates.
18. The method as recited in Claim 17, wherein the membrane containing the I_{Kr} channel is derived from a HEK 293 cell line transfected with the human ERG gene.

19. The method as recited in Claim 8, wherein the radioligand compound of Formula I possesses a specific activity of in the range of about 900 Ci/mmol. to about 1498 Ci/mmol.

5 20. A method for assessing the binding of a test compound to a membrane containing the I_{K_r} channel using a radioligand of Formula I, [^{35}S]-radiolabeled (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxy-2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide, comprising the steps of:

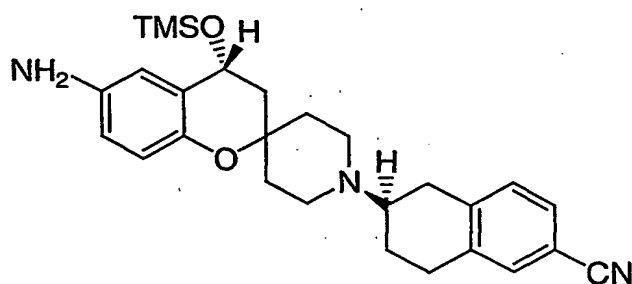
- 10 1) preparing assay wells with 4 μl of the test compound in dimethylsulfoxide (DMSO) diluted 100x with assay buffer at 5 or more different concentrations, a control vehicle of DMSO and a DMSO solution of (+)-N-[1'-(6-cyano-1,2,3,4-tetrahydro-2(R)-naphthalenyl)-3,4-dihydro-4(R)-hydroxy-2H-1-benzopyran-2,4'-piperidin]-6-yl]-methanesulfonamide (compound of Formula II);
- 15 2) adding the radioligand compound of Formula I at 50pM to the membrane containing the I_{K_r} channel diluted with assay buffer to form a membrane/radioligand mixture;
- 20 3) incubating each assay well with 400 μl of the 50 pM membrane/radioligand mixture for about 75 minutes to about 90 minutes at room temperature (25°C) to give assay wells containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II where the final concentration of the membrane containing the I_{K_r} channel is 11 $\mu\text{g}/\text{ml}$;
- 25 4) filtering the incubated assay wells through 0.1% BSA presoaked filters to isolate on the filters the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II;
- 30 5) washing each of the filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II about 5 times with 500 μl of ice cold wash buffer;

- 6) drying the washed-filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II at room temperature in a fume hood;
- 5 7) adding 50 μ l Microscint-20 microscintillate to the dried-filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II;
- 10 8) measuring the microscintillation count of the microscintillation-treated filters containing the membrane bound with the radioligand and the test compound at each concentration, the DMSO control vehicle or the compound of Formula II for one minute; and
- 15 9) calculating the IC₅₀ corresponding to the measured microscintillation count of: 1) the microscintillation-treated filters containing the membrane bound with the radioligand and each concentration of the test compound, 2) the microscintillation-treated filters containing the membrane bound with the radioligand and with the control vehicle, and 3) the microscintillation-treated filters containing the membrane bound with the radioligand and the compound of Formula II.
- 20
21. The method as recited in Claim 20, wherein the membrane containing the I_{Kr} channel is derived from a cell line transfected with the ERG gene.
- 25 22. The method as recited in Claim 21, wherein the cell line is HEK 293 cells.
23. The method as recited in Claim 22, wherein the ERG gene is human or canine.
- 30 24. The method as recited in Claim 23, wherein the solutions of the test compound are prepared in Step 1 at 7 different concentrations.
25. The method as recited in Claim 24, wherein the membrane containing the I_{Kr} channel is derived from a HEK 293 cell line transfected with the
- 35 human ERG gene.

26. The method as recited in Claim 25, wherein the radioligand compound of Formula I possesses a specific activity of in the range of about 900 Ci/mmol. to about 1498 Ci/mmol.

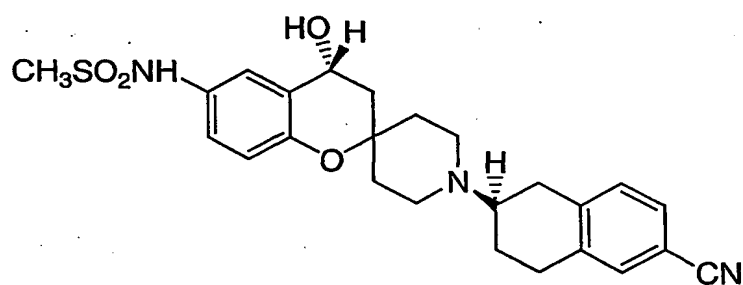
27. The method as recited in Claim 26, wherein the membrane-bound with radioligand or test compound is filtered in Step 4 with Unifilters.

28. A process for the preparation of

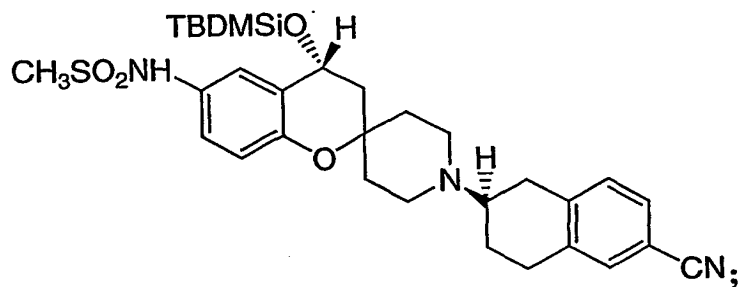


comprising the steps of

(1) reacting the alcohol with 2,6-lutidine and t-butyldimethylsilyl triflate

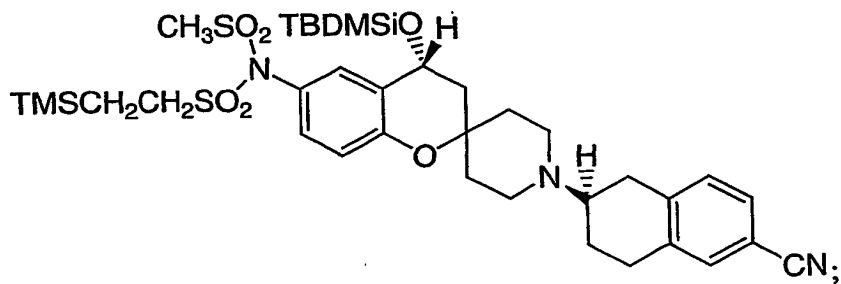


to give a t-butyldimethylsilyl-protected hydroxyl compound



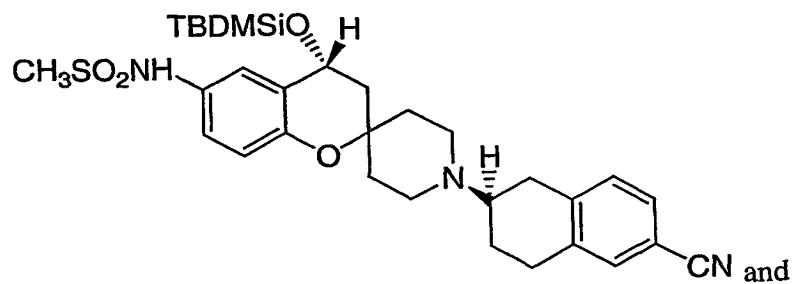
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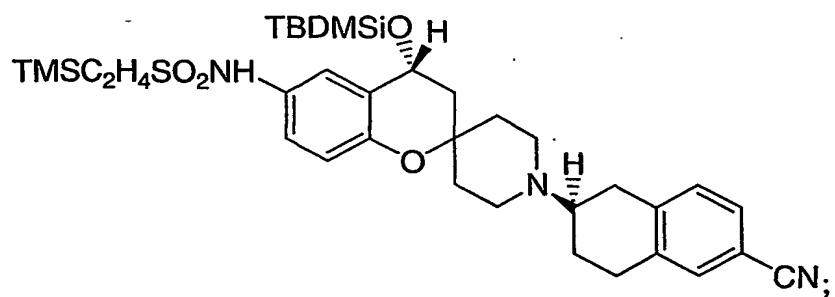
(2) alkylating the t-butyldimethylsilyl-protected hydroxyl compound by treating with sodium hydride, and then treating with 2-trimethylsilylethanesulfonylchloride to give the disubstituted sulfonamide compound



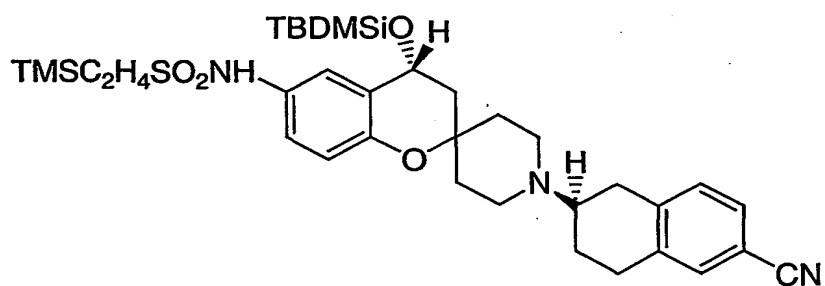
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(3) treating the disulfonamide with an C₁-C₈-alkanethiolate to give a mixture of the following sulfonamides;



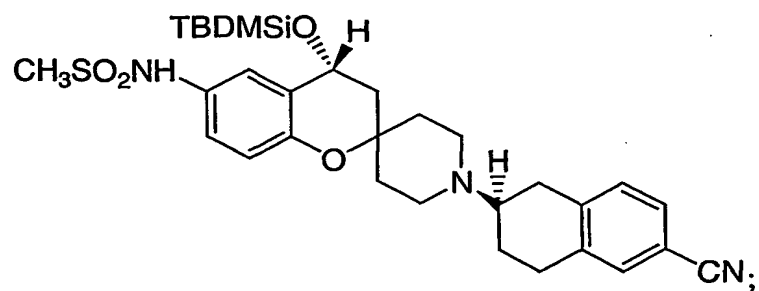


(4) separating the sulfonamide mixture using chromatography to isolate the non-polar sulfonamide eluting with a non-polar solvent system

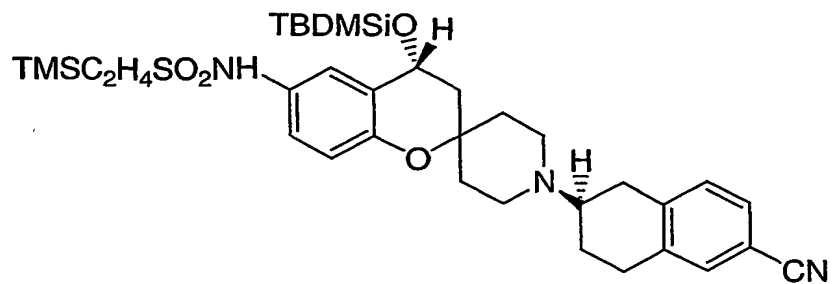


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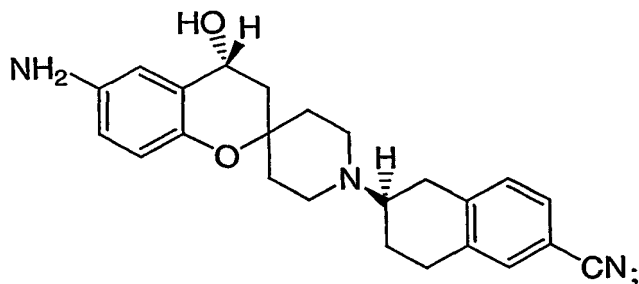
and then eluting off the polar isomer using a polar solvent system



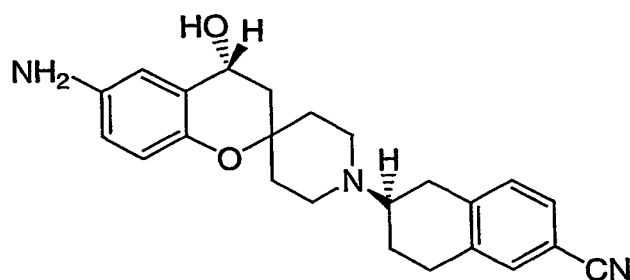
(5) desulfonating the non-polar isomer



using a fluoride compound in an organic base and heating for about 24 hours to about 48 hours to give the free alcohol-amine



- 5 (6) reacting the free alcohol-amine with trimethylsilyl imidazole in an organic solvent



to give the desired trimethylsilyloxy compound.

- 10 29. The process as recited in Claim 28, wherein the alkylation in step 2 is stirred at room temperature for up to 24 hours.
30. The process as recited in Claim 29, wherein the C₁-C₈-alkanethiolate in step 3 is sodium methanethiolate, sodium ethanethiolate, sodium
- 15 propanethiolate or sodium 2-methylpropanethiolate.
31. The process as recited in Claim 30, wherein the desulfonylation reaction in step 3 is run for less than 24 hours.

32. The process as recited in Claim 31, wherein the separation of the sulfonamide mixture in step 4 is run using flash chromatography with a solvent system of ethyl acetate and hexane to ethyl acetate and methanol.

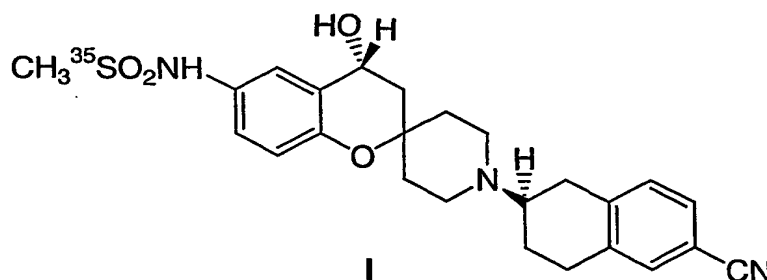
33. The process as recited in Claim 32, wherein the separation of the sulfonamide mixture in step 4 is run using flash chromatography with a non-polar solvent system of 1:1 ethyl acetate: hexane to a polar solvent system of 99:1 ethyl acetate: methanol.

34. The process as recited in Claim 33, wherein The fluoride compound used in the desulfonylation of step 5 is selected from: cesium fluoride and tetrabutylammonium fluoride.

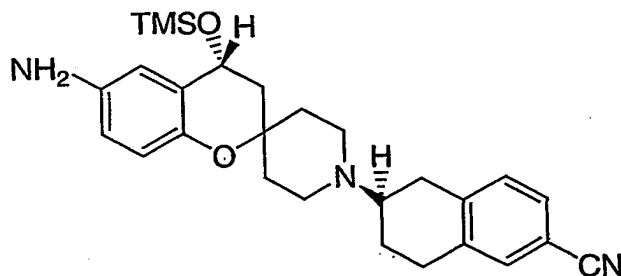
35. The process as recited in Claim 34, wherein the organic solvent used in the desulfonylation of step 5 is selected from: dimethylformamide, dimethylsulfoxide and N-methylpyrrolidinone.

36. The process as recited in Claim 35, wherein the organic solvent in step 6 is acetonitrile, tetrahydrofuran, or ether.

37. A process for the preparation of a radioligand compound of Formula I



comprising the steps of:
(a) reacting the amine



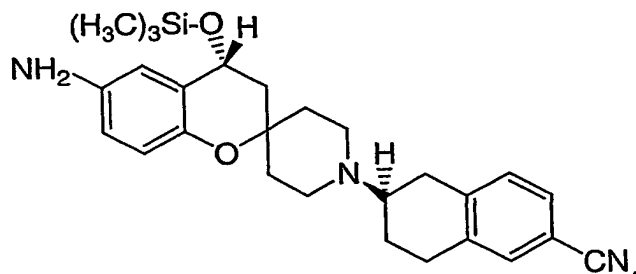
with [^{35}S]-methanesulfonyl chloride in the presence of an organic base to form the silyl-protected [^{35}S]-methanesulfonamide; and

- 5 (b) removing the silyl-protecting group of the silyl-protected [^{35}S]-methanesulfonamide with trifluoroacetic acid to give the radioligand compound of Formula I.

38. The process as recited in Claim 37, wherein the organic base is
10 selected from triethylamine, trimethylamine, and diisopropylethylamine.

39. The process as recited in Claim 38, wherein the organic base is triethylamine.

15 40. A compound which is



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/21731

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 51/04, 31/438 ; G01N 33/567 ; C07D 491/107
US CL : 424/1.81 ; 436/504 ; 546/14, 17 ; 514/278

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.81 ; 436/504 ; 546/14, 17 ; 514/278

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y - A	WO 95/23146 A1 (MERCK & CO., INC.) 31 August 1995 (31.08.95), page 1, lines 15-23.	1-27 ----- 28-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 SEPTEMBER 2001

Date of mailing of the international search report

31 DEC 2001

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